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RESEARCH ARTICLE

HID-1 is a peripheral membrane protein primarily associated with the *medial*- and *trans*-Golgi apparatus

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ABSTRACT

Caenorhabditis elegans hid-1 **gene was first identified in a screen for mutants with a high-temperature-induced dauer formation (Hid) phenotype. Despite the fact that the *hid-1* gene encodes a novel protein (HID-1) which is highly conserved from *Caenorhabditis elegans* to mammals, the domain structure, subcellular localization, and exact function of HID-1 remain unknown. Previous studies and various bioinformatic softwares predicted that HID-1 contained many transmembrane domains but no known functional domain. In this study, we revealed that mammalian HID-1 localized to the *medial*- and *trans*-Golgi apparatus as well as the cytosol, and the localization was sensitive to brefeldin A treatment. Next, we demonstrated that HID-1 was a peripheral membrane protein and dynamically shuttled between the Golgi apparatus and the cytosol. Finally, we verified that a conserved N-terminal myristoylation site was required for HID-1 binding to the Golgi apparatus. We propose that HID-1 is probably involved in the intracellular trafficking within the Golgi region.**

KEYWORDS HID-1, Golgi, peripheral membrane protein, fluorescent recovery after photobleaching, N-myristoylation

INTRODUCTION

When exposed to harsh environmental conditions, the nematode *Caenorhabditis elegans* (*C. elegans*) forms arrested third stage larvae called dauer (Cassada and Russell, 1975; Riddle, 1997; Fielenbach and Antebi, 2008). Entry into the dauer stage depends on the integration of three external environmental parameters: ambient temperature, food availability, and population density (Riddle, 1997; Fielenbach and Antebi, 2008). Internally, dauer formation is regulated by at least three metabolic signaling pathways (Riddle, 1997; Ailion and Thomas, 2003; Fielenbach and Antebi, 2008). The first is cyclic GMP signaling pathway in sensory neurons that responds to pheromone (Birnby et al., 2000). The second is transforming growth factor-beta (TGF-β) signaling pathway, in which release of a TGF-β-like ligand from sensory neurons leads to activation of receptors and the downstream pathways in target cells (Fielenbach and Antebi, 2008). The third is insulin signaling pathway which plays critical roles in different tissues (Apfeld and Kenyon, 1998).

The *C. elegans hid-1* gene was first identified, by Ailion and Thomas, from a screen for mutants with high-temperature-induced dauer formation (Hid) phenotype (Ailion and Thomas, 2003). The *hid-1* gene encodes a highly conserved protein (HID-1) with a single homolog in *Drosophila melanogaster* (NM_136916), *mouse* (NM_175454) and *Homo sapiens* (NM_030630). Previous studies (Ailion and Thomas, 2003) and several bioinformatic softwares predicted that HID-1 contained many transmembrane domains but no known functional domain. Interestingly, the Hid phenotype of *hid-1* mutants is strongly suppressed by mutations in *daf-16* (Ailion and Thomas, 2003), a transcription factor downstream of insulin signaling, suggesting a possible action of HID-1 in the

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insulin branch of dauer pathway (Inoue and Thomas, 2000a, b). The exact cellular function of HID-1 is unclear but was hypothesized to be involved in vesicular exocytosis.

To explore the exact molecular mechanism of HID-1, we carried out the current study that presents the first picture of the expression pattern, subcellular localization and several molecular properties of mammalian HID-1. Our findings show that HID-1 was highly expressed in secretory cell lines and Golgi-like localization in INS-1 cells. Next, HID-1 localized to the *medial*- and *trans*-Golgi apparatus as well as the cytosol, and the localization was sensitive to brefeldin A (BFA). Furthermore, we provided strong evidences that Golgi-localized HID-1 was a peripheral membrane protein and dynamically shuttled between the Golgi apparatus and the cytosol, in contrast to a putative transmembrane protein. Finally, we demonstrated that a conserved N-terminal myristoylation site was required for HID-1 binding to the Golgi apparatus.

RESULTS

HID-1 expresses in secretory cell lines

First, we tested the expression pattern of HID-1 in several cell lines, including 293ET, HeLa, CHO, PC12, INS-1, and 3T3-L1, using the hHID-1 antiserum (Yu et al., 2011). As shown in Fig. 1A, endogenous HID-1 was only detected in PC12 and INS-1 cells. The results suggest that HID-1 is highly expressed in secretory cell lines, in accordance with neurosecretory functions predicted in *C. elegans* (Ailion and Thomas, 2003). In addition, to determine the subcellular localization of HID-1, we first generated a C-terminal enhanced green fluorescent protein (EGFP)-tagged recombinant protein (hHID-1-EGFP). We found this construct had the same localization with hHID-1 antiserum in INS-1 cells by immunofluorescence (data not shown), and verified that C-terminal EGFP-tagged HID-1 protein could rescue the Hid phenotype in *hid-1* mutant in *C. elegans* (Yu et al., 2011), suggesting the fusion protein is functional. hHID-1-EGFP in INS-1 cells transfected showed different expression levels: cytosol and Golgi-like patterns in higher expression levels whereas clear Golgi-like patterns in lower expression levels in certain condition (Fig. 1B), most of which exhibited a perinuclear structure reminiscent of the Golgi complex, as well as a weak dispersed cytosolic distribution.

HID-1 primarily localizes to the *medial*- and *trans*-Golgi apparatus in INS-1 cells

We further characterized the precise localization using different Golgi markers that label the *cis*-Golgi (GM130) (Nakamura et al., 1995), the *medial*-Golgi/*trans*-Golgi network (Golgi-EGFP) (Gleeson et al., 1994; Yamaguchi and Fukuda, 1995) and the *trans*-Golgi network (TGN38-EGFP) (Luzio et al., 1990; Humphrey et al., 1993). As shown in the upper panels of the Fig. 2A and B, hHID-1 co-localized nicely with Golgi-EGFP, mostly with TGN38-EGFP, and much less with GM130, suggesting that hHID-1 was primarily localized to the *medial*- and *trans*-Golgi apparatus.

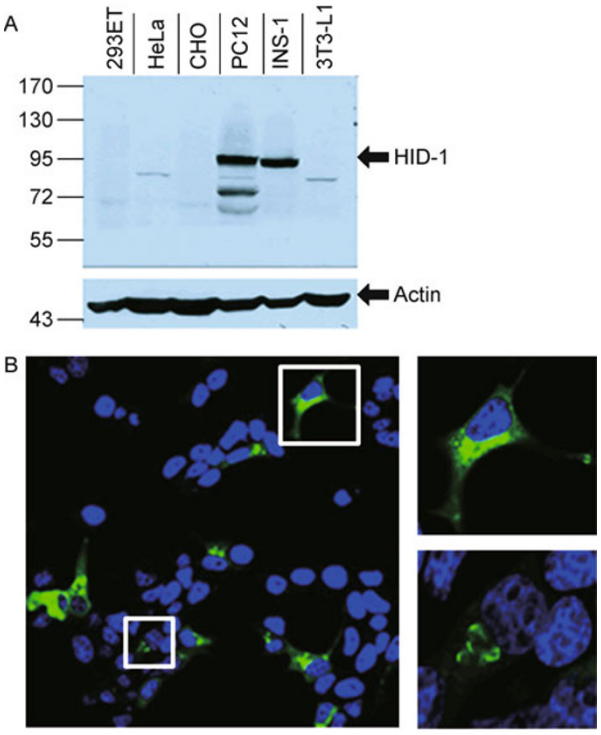

Figure 1. Expression of HID-1 in different cell lines and localization of HID-1 in INS-1 cell line. (A) The expression of HID-1 in different cell lines. Lysates of 293ET, HeLa, CHO, PC12, INS-1 and 3T3-L1 cells were immunoblotted with the hHID-1 antiserum and an anti-actin antibody. Actin was detected by an anti-actin antibody as a loading control. The positions of HID-1 and actin bands were indicated by arrows. (B) After hHID-1-EGFP was transfected in INS-1 cells for 48 h, the cells were imaged for a smaller view on the left side. Different expression levels of the transfectant were enlarged on the right for a better view. Nuclei were labeled with Hoechst 33342 (blue).

Furthermore, we found that the Golgi-localization of HID-1 was sensitive to BFA, a Golgi-disturbing agent (Lippincott-Schwartz et al., 1991). As shown in lower panels of the Fig. 2A and 2B, after 10 μM BFA treatment, exogenous hHID-1-EGFP mostly exhibited a dispersed distribution with some puncta structures. Moreover, part of the hHID-1-EGFP puncta structures co-localize with Golgi-EGFP but not with GM130. Similarly, the Golgi-localization of endogenous rHID-1 was also abolished after BFA treatment and partial co-localization was observed with the TGN marker, TGN46 (Fig. 2C), which is the human homology of rat TGN38 (Ponnambalam et al., 1996).

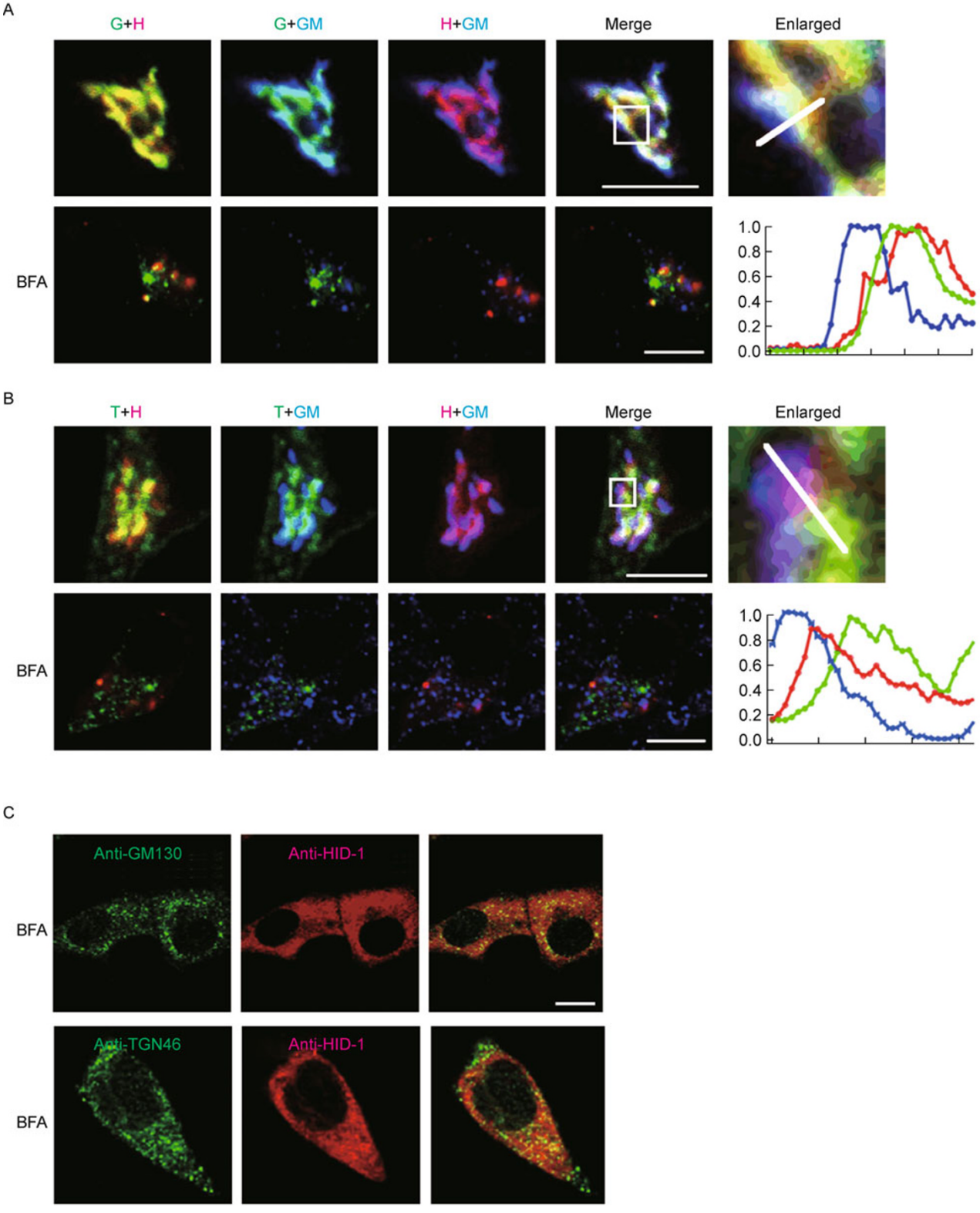

Figure 2. hHID-1 distributes primarily to the *medial*- and *trans*-Golgi apparatus. (A and B) INS-1 cells were co-transfected with hHID-1-mKO and Golgi-EGFP (A) or hHID-1-mKO and TGN38-EGFP (B), and then either untreated (upper panel) or treated with 10 μM BFA (lower panel). Boxed images were enlarged for a better view. The fluorescence intensity linescan profiles were generated along the white lines indicated in the enlarged images. G: Golgi-EGFP (*medial*- and *trans*-Golgi marker), H: hHID-1-mKO, GM: GM130 (*cis*-Golgi marker, endogenous, Cy5 used as the secondary antibody), T: TGN38-EGFP (*trans*-Golgi marker). Scale bar = 10 μm. (C) INS-1 cells were treated with BFA for 30 min and then immunofluorescent labled with an anti-GM130 antibody, an anti-TGN46 antibody, and the hHID-1 antiserum respectively. Scale bar = 10 μm.

A portion of HID-1 distributes to the cytosol

Besides the predominant signals in the Golgi network, visible fluorescent signals were observed in the cytosol region using exogenous hHID-1-EGFP expression and immunofluorescence analysis of endogenous rHID-1 in INS-1 cells (Yu et al., 2011). To validate the cytosolic distribution of HID-1, we developed a novel method to determine the cytosolic proteins in living cells based on rapamycin-mediated heterodimerization (Choi et al., 1996) of FKBP (FK506 binding protein) (Standaert et al., 1990) and FRB (mTOR) (Brown et al., 1994). We first tagged FKBP to the C-terminus of hHID-1-EGFP, and then transfected it together with lyn11 (plasma membrane marker)-FRB (Suh et al., 2006) and Golgi-TDimer2 into INS-1 cells. As shown in Fig. 4, hHID-1-EGFP-FKBP primarily co-localized with Golgi-TDimer2 in control cells without rapamycin. After addition of rapamycin, weak fluorescent signals of hHID-1-EGFP-FKBP in the cytosol disappeared and distinct signals were observed at the plasma membrane, whereas no apparent change was observed for the transmembrane Golgi marker, Golgi-TDimer2. The results assured the partial localization of HID-1 to the cytosol. The method we developed here provides a robust alternative to determine whether a protein actually expresses in the cytosol, as opposed to the classic biochemical ultra-centrifugation assays (Nakamura et al., 1995; Liang and Li, 2000; Luo et al., 2008).

HID-1 is a peripheral membrane protein

The hydropathy plot of HID-1 protein was analyzed by various softwares to predict its transmembrane domain(s). Several softwares, including TMpred, HMMTOP2.0, DAS, and Toppred2, predicted multiple transmembrane domains in HID-1. The results suggest that HID-1 contains two highly hydrophobic domains from amino acids 416 to 437 and 509 to 532; however, both of them failed to reach the threshold set by TMHMM2.0.

To address whether HID-1 is a transmembrane protein, we employed both an *in vitro* biochemical membrane fraction analysis (Nakamura et al., 1995; Liang and Li, 2000; Luo et al., 2008) and an *in vivo* permeabilization assay in living cells (Lorenz et al., 2006; Dimitrov et al., 2009). In the biochemical assay, INS-1 cells were ultracentrifuged to obtain the cytosolic fraction (the supernatant, S) and the membrane fraction (the pellet, P). Solutions with high salt, high pH, and 1% Triton X-100 were used to extract proteins from the membrane fraction, respectively. The different fraction samples were immunoblotted with anti-hHID-1 antiserum and anti-Syntaxin 1A antibody. As shown in Fig. 5, rHID-1 was primarily detected in the membrane fraction and also observed in the cytosol, which was consistent with the cytosolic expression determined by the rapamycin-based method we developed (Fig. 4). Notably, treatment with 0.1 M Na_2CO_3 (pH 11.5) caused rHID-1 to be released from the membrane fraction into the soluble fraction; however, 1 M NaCl and 1% Triton X-100 solution did not cause its release. As a control, the integral membrane protein marker Syntaxin 1A (Koh et al., 1993; Lewis et al., 2001; Trus et al., 2001) failed to dissociate into the supernatant in high salt or high pH solutions, but dissolved in 1% Triton X-100 solution. Another control, an integral membrane protein transferring receptor (Zerial et al., 1986; Campellone et al., 2008), which is cycled between the plasma membrane and early endosome (Killisch et al., 1992), was also used, and the same distribution with Syntaxin 1A was observed (data not shown). The results suggest that HID-1 is a peripheral membrane protein tightly associated with the membrane.

To further determine the peripheral Golgi localization of HID-1, we adopted a permeabilization assay using a high concentration of digitonin according to a previously described protocol (Lorenz et al., 2006; Dimitrov et al., 2009). Digitonin has cholesterol-binding ability that can effectively permeabilize the plasma membrane without perturbing the structure of the Golgi apparatus. In this assay, we utilized a single transmembrane *trans*-Golgi marker TGN38-EGFP (Humphrey et al., 1993) as an integral membrane protein control and simultaneously recorded fluorescence with hHID-1-mKO before and after adding a high concentration of digitonin (60 μM). As shown in Fig. 6, the fluorescence of hHID-1-mKO disappeared over time after adding digitonin; however the fluorescence of TGN38-EGFP only decreased slightly probably due to photobleaching. Together with the biochemical assay, the results strongly demonstrate that HID-1 is a peripheral membrane protein tightly associated with the Golgi apparatus but not a transmembrane protein predicted by the bioinformatic programs.

HID-1 shuttles dynamically between the Golgi apparatus and the cytosol

Being a peripheral membrane protein, HID-1 should shuttle between the Golgi apparatus and the cytosol. We analyzed the dynamic rate of hHID-EGFP in INS-1 cells by fluorescence recovery after photobleaching (FRAP) approach. A reported Golgi anchoring protein, Dymeclin, was used as a control, which is known to be a fast shuttling protein between the Golgi apparatus and the cytosol (Dimitrov et al., 2009). When EGFP-tagged Dymeclin was co-transfected with hHID-1-mKO in INS-1 cells, we observed perfect co-localization of the two proteins (Fig. 7A). This co-localization was even maintained after disrupting the Golgi apparatus with BFA. The recovery of Dym-EGFP (EGFP-tagged at the C-terminus of Dymeclin) and HID-1-EGFP were then recorded by consecutive imaging after the photobleaching. As shown in Fig. 7B, the representative FRAP images of hHID-1-EGFP demonstrated that cytosolic hHID-1 could shuttle to Golgi membrane. The mobile fraction of hHID-1-EGFP only

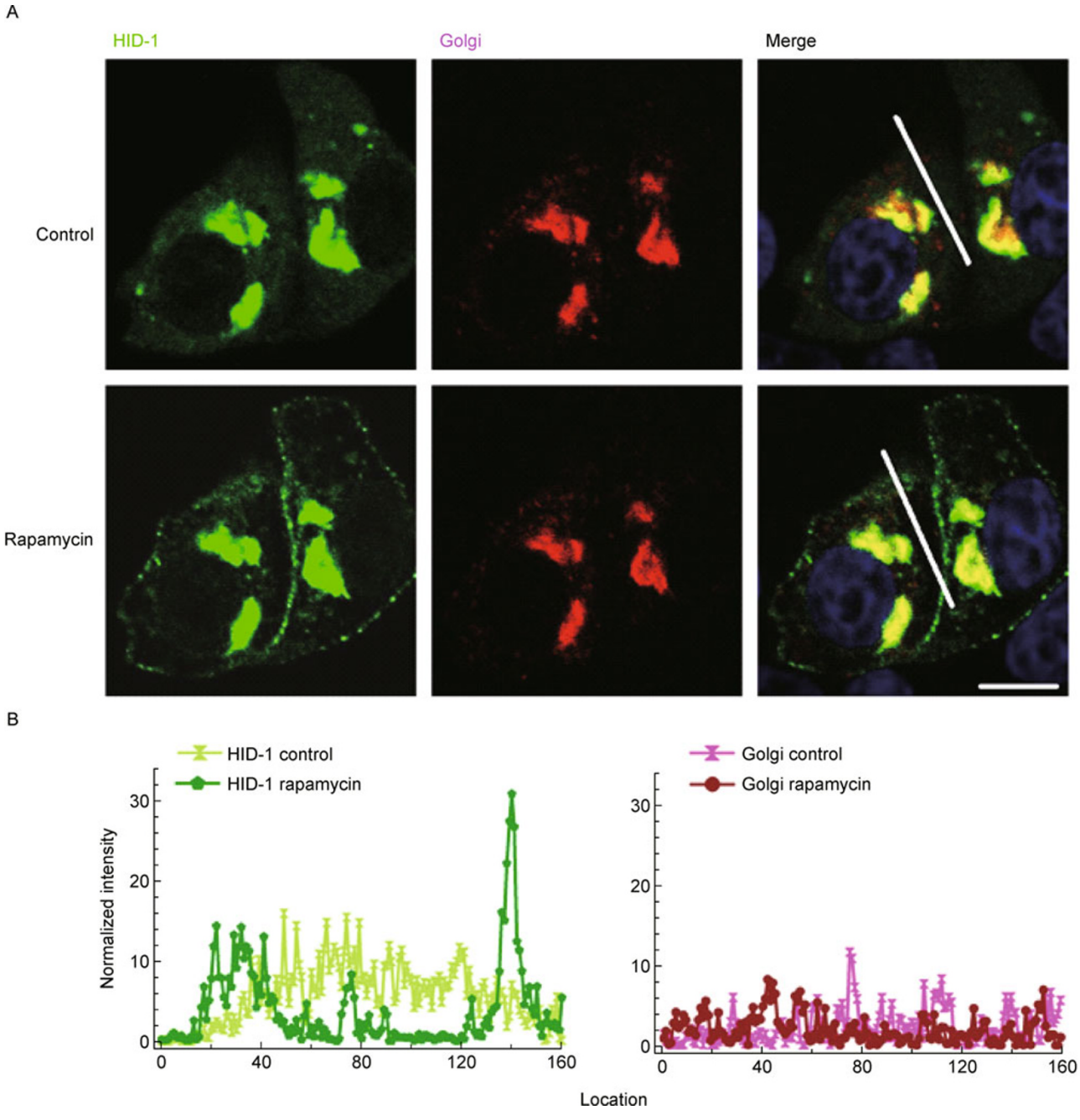

Figure 3. Demonstration of cytosolic portion of HID-1 by rapamycin-mediated heterodimerization of FKBP and FRB. (A) INS-1 cells were transfected with hHID-1-EGFP-FKBP, Golgi-TDimer2 and lyn11-FRB together. hHID-1-EGFP-FKBP and Golgi-TDimer2 were observed to co-localize at the Golgi region (upper panel). After rapamycin treatment for 2 min, hHID-1-EGFP-FKBP was observed at the plasma membrane, whereas the localization of Golgi-TDimer2 was not affected. Scale bar = 10 μm. (B) The fluorescence intensity linescan profiles were generated along the white lines indicated in (A), suggesting the redistribution of hHID-1-EGFP-FKBP from cytosol to the plasma membrane after rapamycin treatment.

restored a small portion of the pre-bleaching fluorescent level in the Golgi region, consistent with the idea that HID-1 preferentially localizes to the Golgi apparatus. The half-time recovery of hHID-1-EGFP was calculated to be 16.4 ± 0.1 s (n = 23), while Dym-EGFP had a faster dynamics with a half-time of 5.3 ± 0.1 s (n = 17) (Fig. 7D). These results suggest that HID-1 dynamically shuttles between the Golgi apparatus and the cytosol.

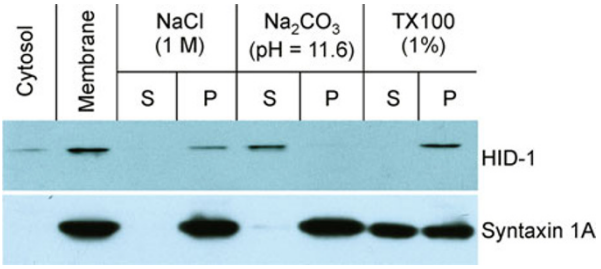

Figure 4. HID-1 is a peripheral membrane protein. After separation from the cytosol, INS-1 membrane fractions were treated with 1 M NaCl, 100 mM Na_2CO_3 (pH 11.5), or 1% Triton X-100 for 30 min at 4°C respectively, followed by ultra-speed centrifugation. The supernatants (S) and pellets (P) in different samples were detected by immunoblot with the hHID-1 antiserum and an anti-Syntaxin1A antibody.

N-myristoylation of HID-1 plays a key role in its Golgi-localization

Although no conserved domains or signal peptides could be predicted from the HID-1 primary sequence, a putative N-terminal myristoylation site "MGSTDSKLN" was predicted by the "Myristoylator" software (prediction of N-terminal myristoylation by neural networks, NMT). Protein N-myristoylation corresponds to the covalent attachment of a myristate, a 14-carbon saturated fatty acid, to the N-terminal glycine of eukaryotic proteins and promotes a weak and reversible membrane association in many cases. N-myristoylation plays key roles in critical components of various cellular processes, such as signal transduction, apoptosis, and oncogenesis (de Jonge et al., 2000; Wright et al., 2009). To test the function of the predicted myristoylation site in HID-1, we mutated the amino acid "G" at position + 2 of hHID-1-mKO to a nonfunctional amino acid "A" (hHID-1(G2A)-mKO) and examined its co-localization with hHID-1-EGFP in living cells by confocal imaging. As shown in Fig. 8, in INS-1 cells hHID-1(G2A)-mKO detached from the Golgi apparatus and appeared to have a diffused cytosolic distribution, in contrast to the Golgi-localization of hHID-1-EGFP in the same cell. The results indicate that the predicted N-myristoylation site is required for HID-1 binding to the Golgi apparatus.

DISCUSSION

The function of a given protein depends on the local environment where the protein resides. In this respect, it is crucial to determine the precise subcellular localization of a protein before concluding on its function. Various programs have predicted HID-1 protein as a transmembrane protein. In contrast to these predictions, we have verified that HID-1 is a peripheral membrane protein that associates primarily with the *medial*- and *trans*-Golgi membranes. Peripheral membrane proteins are associated with the surface of the membrane by their conformational structure (such as amphipathic helices, hydrophobic loops) and/or electrostatic interactions (involved with lipid-protein and/or protein-protein interactions) which can be disrupted by high concentration of salt solution, high pH buffers or metal chelating agents. Indeed, we found high pH (pH 11.5) buffer released HID-1 from the membrane fraction to soluble fraction (Fig. 5). In addition, we demonstrated that HID-1 dissociated from the Golgi membrane upon permeabilization of plasma membrane by digitonin, in contrast to an integral transmembrane Golgi marker, TGN38 (Fig. 6). These results demonstrate a membrane anchoring nature of HID-1 and suggest a cytosolic portion of HID-1 protein.

To further validate the cytosolic existence of HID-1, we established an original method based on rapamycin-mediated heterodimerization of FKBP and FRB. In recent years, the advantages of the FKBP/FRB/rapamycin system (Standaert et al., 1990; Brown et al., 1994; Choi et al., 1996) have been shown by various kinds of significant applications including the localization and activity of Golgi-resident enzymes such as glycosyltransferase (Dube et al., 2006), and the rapid inactivation of endogenous proteins by specific siRNA-resistant sequence tagged FKBP (or FRB) (Robinson et al., 2010). Here, we have expanded the application of FKBP/FRB/rapamycin complex, providing a reliable shortcut to judge whether a certain protein has a cytosolic fraction. This method is robust as we observed rapid translocation of FKBP-tagged hHID-1 to the plasma membrane with concomitant reduction in cytosolic portion (Fig. 4). The existence of two pools of HID-1 proteins prompts us to speculate whether HID-1 can dynamically shuttle between the cytosol and the Golgi membrane, with a preference to the membrane. This speculation is in agreement with our FRAP experiments (Fig. 7) where we showed that cytosolic HID-1 could associate with Golgi membrane with a half-time of ~16 s. The association with Golgi membrane seems to be mediated primarily by N-myristolylation of a glycine residue at the N-terminal end. The functions of a large number of Golgi proteins with N-myristoylation have already been established. For example, Arfs and GRASPs (Mironov and Pavelka, 2008) (such as GRASP55 and GRASP65) proteins are all targeted to the membrane by N-myristoylation (Lodge et al., 1997). Therefore, HID-1 may use a similar mechanism as these proteins for anchoring the corresponding membranes.

A striking discovery of this study is that HID-1 is a novel Golgi-localized protein with a preference to the *medial*- and *trans*-Golgi apparatus. The subcellular localization of HID-1 resembles its only paralog, Dymeclin (Dimitrov et al., 2009). Dymeclin is a protein recently identified as the translation product of the FLJ20071/FLJ90130/Dym gene (*DYM*), mutations of which are responsible for a severe autosomal-recessive skeletal dysplasia associated with mental delay, Dyggve–Melchior–Clausen syndrome (DMC, MIM#223800) and a clinical variant without mental

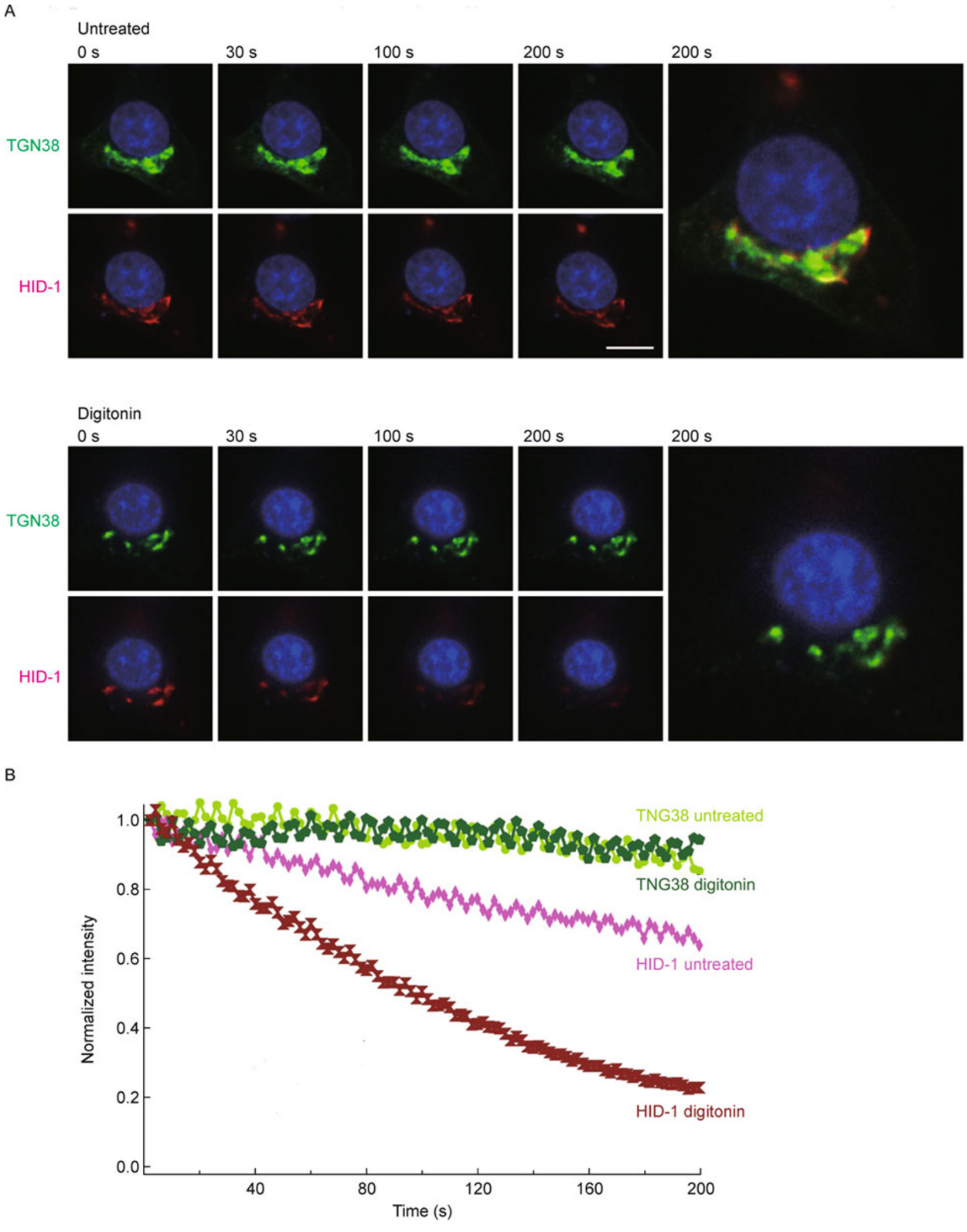

Figure 5. HID-1 dissociates from the Golgi membrane after the permeabilization of digitonin. (A) INS-1 cells were transfected with hHID-1-mKO (red) and TGN38-EGFP (green). Then imaging was performed on a spinning disk microscope before (control) and after digitonin treatment. Nuclei were labeled with Hoechst 33342 (blue). Scale bar = 10 μm. (B) Normalized fluorescent intensity plots of Golgi region at indicated time.

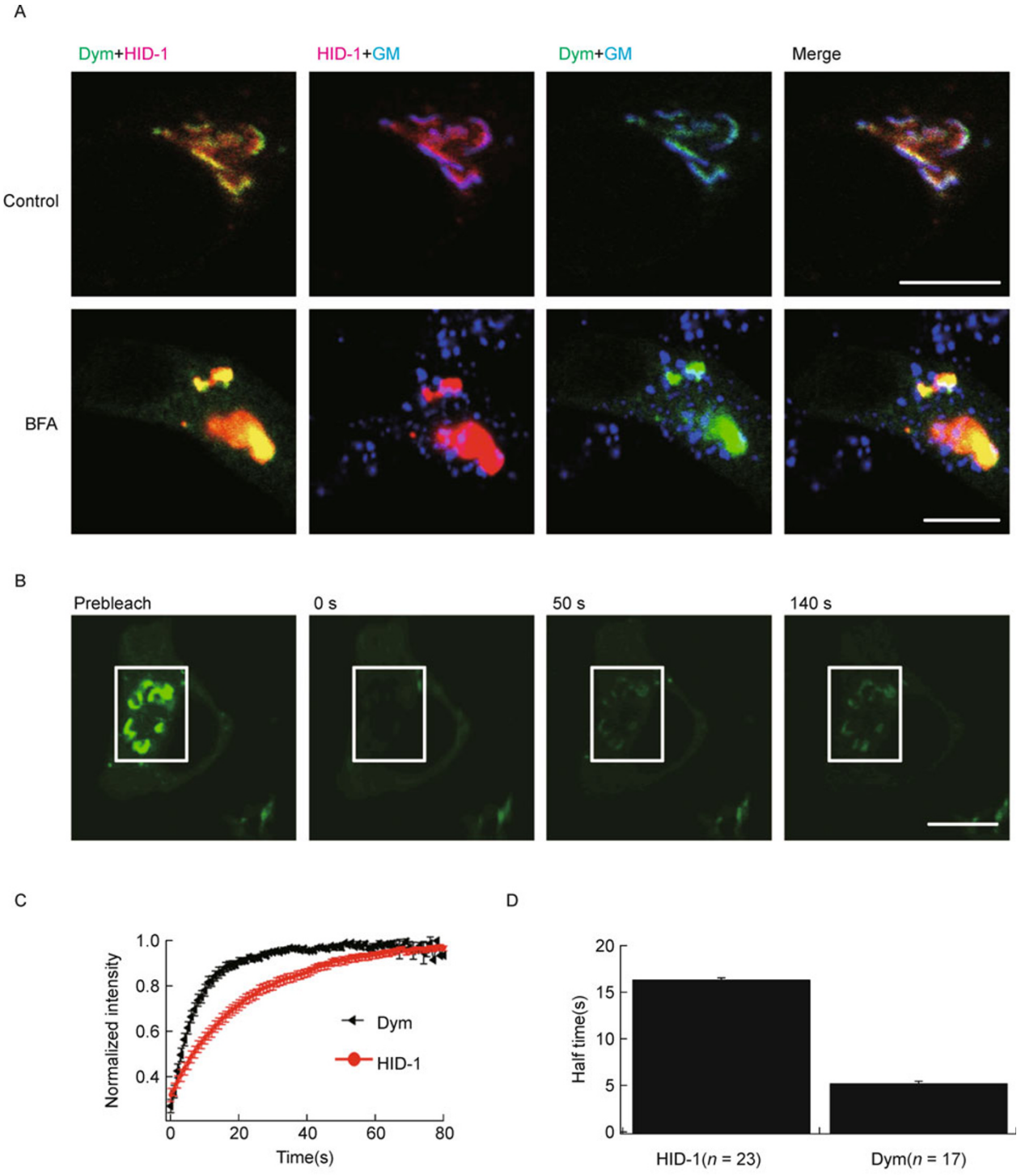

Figure 6. Dynamics of hHID-1 and Dymeclin on Golgi apparatus in INS-1 cells by FRAP approach. (A) Localization of hHID-1 and Dymeclin in INS-1 cells. Dym (Dym-EGFP, transfected); HID-1(hHID-1-mKO, transfected). GM: GM130 (*cis*-Golgi marker, endogenous, Cy5 used as the secondary antibody). Scale bar = 10 μm. (B) hHID-1-EGFP transiently expressed in INS-1 cells was imaged before and after photobleaching the Golgi region (framed by the rectangle) at different time points by Olympics FV1000 confocal microcopy. (C) Normalized fluorescent intensity recovery curve after photobleaching of Dym-EGFP and hHID-1-EGFP. (D) Quantified half recovery time of hHID-1-EGFP (16.4 ± 0.1 s, $n = 23$) and Dym-EGFP (5.3 ± 0.1 s, $n = 17$).

impairment, Smith–McCort syndrome (SMC, MIM#607326) (Cohn et al., 2003; El Ghouzzi et al., 2003). Dymeclin is a 669-

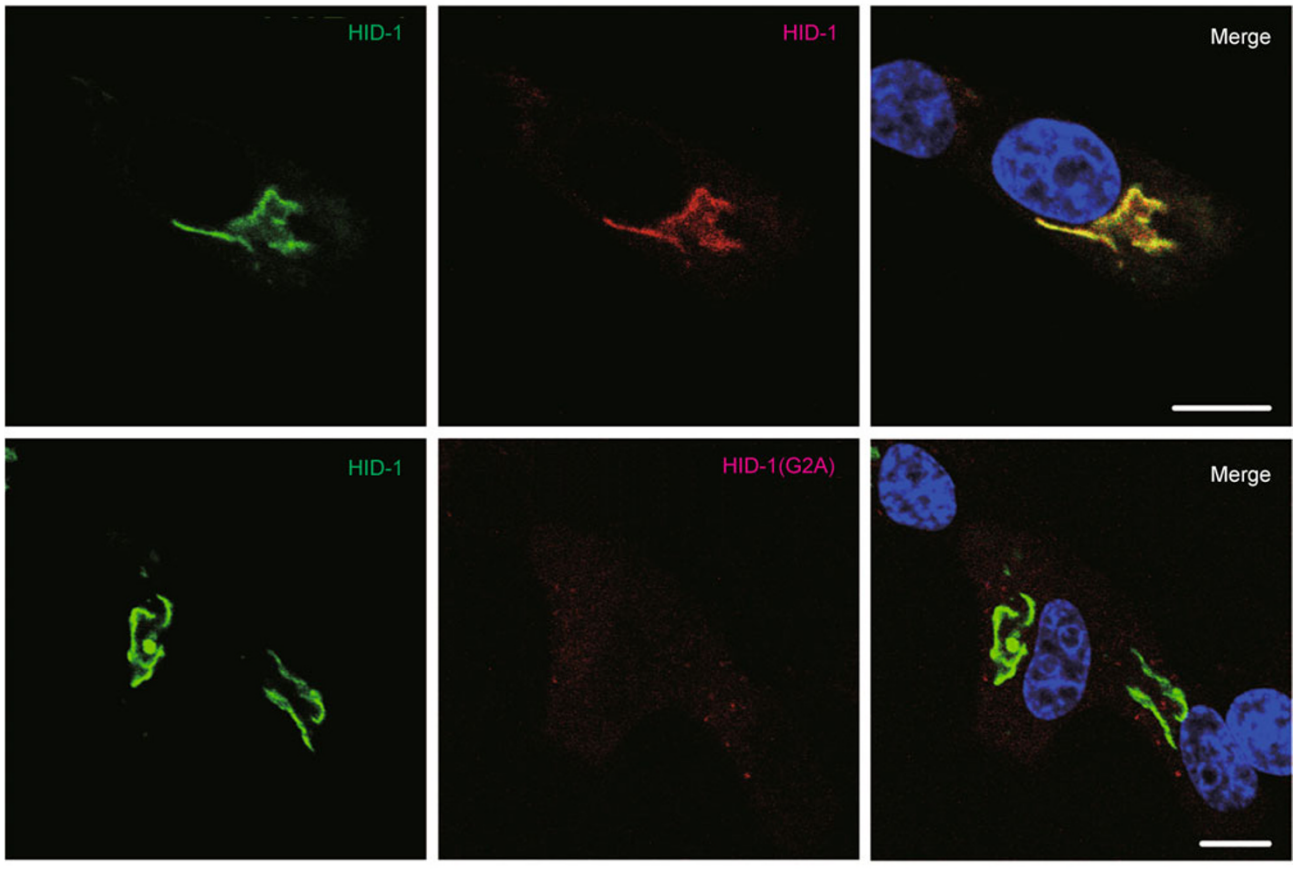

Figure 7. HID-1 anchors to the Golgi membrane by N-myristoylation. The upper panel shows that the localization of co-tranfected hHID-1-EGFP (green) and hHID-1-mKO (red) in INS-1 cells, and the lower panel shows that co-transfected hHID-1-EGFP (green) and hHID-1(G2A)-mKO (red) in the same batch of INS-1 cells. Nuclei were labeled with Hoechst 33342 (blue). Scale bar = 10 μm.

amino acid protein which does not belong to any identified protein family. Except for a high homology with HID-1 protein, search for predictive structural and/or functional motives failed to provide any clue as to the function of Dymeclin (Cohn et al., 2003; El Ghouzzi et al., 2003). Electron microscopy revealed that Dymeclin associated with the Golgi apparatus and transitional vesicles of the reticulum-Golgi interface. In this study, we have shown that HID-1 co-localized pretty well with Dymeclin at Golgi region (upper panel of Fig. 7A). Interestingly, this co-localization even persisted after disrupting the Golgi structure by BFA (lower panel of Fig. 7A). These results suggest that HID-1 and Dymeclin reside on the same subdomain of Golgi apparatus.

The Golgi apparatus serves as a crossroad of intracellular trafficking, including ER-Golgi transport, Golgi-to-plasma membrane traffic, and transport from TGN to endosomes and lysosomes. Recent decades witness an exponential increase of proteins identified as peripheral membrane proteins of the Golgi apparatus, such as SNAPs (soluble NSF attachment proteins) (Colombo et al., 1998) and p230 (Kjer-Nielsen et al., 1999). Many peripheral membrane proteins have been found to be involved in intracellular trafficking in secretory pathway, such as P115 (Waters et al., 1992) and ARFs (Anders and Jurgens, 2008). Based on the subcellular localization, it has been proposed that Dymeclin might be involved in vesicular trafficking to and within Golgi apparatus. Considering previous results in *C. elegans* suggesting a role for *hid-1* gene in the insulin pathway that regulates dauer formation and the current results that HID-1 co-localized with Dymeclin in subdomain of Golgi apparatus, we propose that HID-1 may function in the trafficking of cargos that are important for the sorting/biogenesis/maturation of dense core vesicles (DCVs). Further experiments are definitely in need to unravel the precise function of this mysterious protein.

MATERIALS AND METHODS

Cell culture and transfection

INS-1 cells were cultured in RPMI-1640 (Gibco) with 11.1 mM D-glucose plus 10% fetal bovine serum (Gibco), 1 mM sodium pyruvate

(Sigma), and 50 mM β-mercaptoethanol (Sigma), and were maintained at 37°C and 5% CO_2 in a humidified incubator. Cells were transiently transfected with Lipofectamine TM 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, cells were grown in RPMI-1640 medium for 36 h and then processed.

Plasmids

The full-length *Homo sapiens* HID-1 was isolated from human brain cDNA, and was then cloned into *Nhe* I / *Hin*d III sites of pEGFP-N1. For the construction of hHID1-mKO, EGFP was replaced by mKO, without other changes. The hHID-1(G2A)-mKO mutant was produced through replacement of amino acid "G" at position + 2 of hHID1-mKO with amino acid "A". Full-length of hHID-1 was tagged with FKBP and EGFP sequentially in the hHID-1-FKBP-EGFP plasmid. The *Homo sapiens* Dymeclin purchased from ProteinTech Group was cloned in pEGFP-N1 at *Nhe*I/*Xho*I sites. In addition, Lyn11-FRB was a kind gift from Tobias Meyer (Stanford University, Palo Alto, CA, USA), and TGN38-EGFP was generously provided by Peter A. Greer (Queen's Cancer Research Institute, Canada). Golgi-EGFP ("Golgi" means 81 amino acids of the precursor to the human beta 1, 4-galactosyltransferase) was purchased from Clontech Laboratories, and EGFP was replaced by TDimer2 for the construction of Golgi-TDimer2.

Antibodies

For immunofluorescence, primary antibodies were used as following: anti-hHID-1 antiserum was produced from the blood of mice immunized with purified full-length human HID-1 recombinant protein expressed by AbMax Biotechnology Company (China). Commercial antibodies included mouse anti-Syntaxin1A (Sigma, S0664), mouse anti-FLAG (Sigma, F1804), rabbit anti-Syntaxin6 (Proteintech Group, 10841-1-AP), and mouse anti-GM130 (BD Transduction Laboratories 610822). All the secondary antibodies used for immunofluorescence were from Jackson ImmunoResearch.

Subcellular fractionation for membrane protein extraction

Three 10 mm plates of INS-1 cells were washed twice with 10 mL ice-cold PBS and scraped in 5 mL ice-cold PBS with 1 mM PI (protease inhibitor cocktail). Cells were then homogenized in 5 mL Buffer A (0.3 M sucrose, 1 mM EDTA, 1 mM $MgSO_4$, 10 mM MES-KOH, pH 6.5) by the Dounce homogenizer on ice. The homogenate (whole cell) was centrifuged at 1000 × *g* for 5 min to remove unbroken cells and nuclear debris. The supernatant from the original 1000 × *g* spin was used to separate cytosol from total membranes by centrifugation at 200, 000 × *g* for 30 min at 4°C in a Beckman TLA 100.3 rotor. The resultant pellets were subjected to treatment with different solvents (including 0.1 M Na_2CO_3 at pH 11.5, 1 M NaCl, or 1%Triton X-100) by end-over-end mixing (30 min, 4°C), and then centrifuged at 100,000 × *g* and 4°C for 30 min. The supernatant and pellet were analyzed by Western blotting.

Immunofluorescence and confocal microscopy analysis

Indirect immunofluorescence experiments were performed as previously described with minor modifications. INS-1 cells were cultured onto glass coverslips after transfection and at approximately 36 h post-transfection, and cells were fixed with 4% (*w/v*) paraformaldehyde in PBS for 20 min at room temperature. After three washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, blocked by 5% goat serum, followed by incubation with primary antibodies diluted in PBS for 1 h. Subsequently, cells were washed three times with PBS and incubated with secondary antibodies diluted in PBS for 1 h. After washing three times with PBS, the cells were mounted using anti-fading reagents for imaging.

All confocal microscopy images (except the FRAP assay) were obtained using the Olympus FV500 Laser Scanning Confocal Microscopy and spinning-disk confocal microscopy. Confocal settings used for image capture were held constant in comparison experiments. Images were quantified and analyzed using Image J software (NIH, USA). In the FRAP experiment, hHID-1-EGFP and Dym-EGFP were imaged by Olympus FV1000 Laser Scanning Confocal Microscopy every 3 s at a lower intensity illumination after selective photobleaching with a higher intensity.

Bioinformatics analysis

Prediction of the hydrophobic amino acid and transmembrane regions was performed using the following software programs: TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/), HMMTOP2.0 (http://www.enzim.hu/hmmtop/), DAS (http://www.sbc.su.se/~miklos/DAS/), Toppred2 (http://www.sbc.su.se/~erikw/toppred2/). Post-translational modification predictions were performed with the following program: Myristoylator-Prediction of N-terminal myristoylation by neural networks.

ABBREVIATIONS

BFA, brefeldin A; EGFP, enhanced green fluorescent protein; mKO, monomeric Kushibara Orange; TDimer2, tandem dimer mutant of Dsred; FRAP, fluorescent recovery after photobleaching; DCV, dense core vesicle; TGF-β, transforming growth factor-beta; TGN, *trans*-Golgi network

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